

# Cytokine-inducible CD40 gene expression in vascular smooth muscle cells is mediated by nuclear factor $\kappa$ B and signal transducer and activator of transcription-1

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**Abstract** The interaction of T-lymphocytes expressing the CD40 ligand (CD154) and cells of the vessel wall expressing the corresponding receptor protein (CD40) may play an important role in chronic inflammation including arteriosclerosis. One way of interfering with CD40-CD154 signalling is to prevent CD40 expression, the regulation of which, however, has yet to be elucidated. Therefore, we studied CD40 expression in rat aortic cultured smooth muscle cells. Both CD40 mRNA and protein expression in these cells was markedly enhanced as early as 6 h after exposure to different pro-inflammatory cytokines. Experiments with actinomycin D and subsequent run-on analyses revealed that CD40 expression in response to these cytokines was regulated at the level of transcription. Moreover, electrophoretic mobility shift analyses along with the employment of transcription factor decoy oligodeoxynucleotides demonstrated that tumor necrosis factor  $\alpha$  via nuclear  $\kappa$ B and interferon- $\gamma$  via signal transducer and activator of transcription-1 up-regulate CD40 gene expression in rat aortic cultured smooth muscle cells.

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**Key words:** Transcription factor; Decoy oligodeoxynucleotide; CD154; T-cell; Chronic inflammation

## 1. Introduction

Initially identified as a B-cell surface antigen, CD40 is now considered as a member of the nerve growth factor/tumor necrosis factor (TNF) receptor family [1–3]. In addition to antigen-presenting cells, expression of CD40 has also been documented for a variety of non-immune cells including endothelial cells, fibroblasts and vascular smooth muscle cells [4]. The ligand for CD40 (CD154) is a membrane-bound member of the TNF gene family [2,3] that is expressed by activated CD4<sup>+</sup> T-cells, mast cells, activated platelets [5] and possibly also in endothelial cells at sites of inflammation [4].

CD40-CD154 interactions play a crucial role not only in humoral and cellular immunity, but also in T-cell-mediated inflammatory responses [1–3]. Thus, activation of circulating monocytes by CD154 enhances their expression of pro-inflammatory cytokines, adhesion molecules and MHC class II antigen [6]. In endothelial cells, CD154 up-regulates the expression of integrins and chemokines and, as a consequence, enhances the recruitment and extravasation of circulating monocytes [7,8]. Activated monocytes and endothelial cells,

on the other hand, augment the expression of CD154 on CD4<sup>+</sup> T-cells [9].

It does not come as a surprise, therefore, that the CD40-CD154-mediated interaction between endothelial cells, activated T-cells and circulating monocytes has been suggested to play an important role in the pathogenesis of various autoimmune diseases [1–3] as well as in cardiac allograft rejection [10] and arteriosclerosis [11,12]. Thus far, no antagonists for CD40 are known and there are no reports regarding the regulation of CD40 expression in CD154 target cells. Indeed, only part of the promoter region of the human CD40 gene has been sequenced, but not yet characterized in detail [13], so that it is not known which transcription factors regulate CD40 expression. We hypothesized that by elucidating these regulatory mechanisms, one may devise a strategy (i.e. down-regulation of CD40 expression in CD154 target cells) for therapeutically interfering with CD40-CD154 signalling in the afore-mentioned pathophysiological conditions. Endothelial and smooth muscle cells are the main target cells for activated T-cells in the vessel wall, especially in arteriosclerosis. Therefore, we have investigated in detail the cytokine-inducible expression of CD40 in cultured smooth muscle cells of the rat aorta (raSMC).

## 2. Materials and methods

### 2.1. Cell culture

Smooth muscle cells were isolated from the rat aorta by the explant technique. Aortae (1.5 mm in diameter) were isolated from pentobarbitone-anesthetized male Wistar rats (250–300 g body weight), cleaned under sterile conditions of adherent fat and connective tissue and cut open longitudinally. The endothelium was scraped off with a razor blade, the adventitia removed by using small forceps and the remainder of the blood vessel was cut into segments of approximately 3 mm width. These were incubated in non-coated six well plates in 1 ml Waymouth medium (Gibco BRL, Paisly, UK via Life Technologies GmbH, Karlsruhe, Germany) containing 10% FBS, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, 10 U/ml nystatin, 5 mmol/l HEPES and 5 mmol/l TES until there was a visible outgrowth of cells (usually within less than 1 week). Thereafter, the segments were removed and the culture medium exchanged every 2 days for approximately 1 week until the cells reached confluence. They were harvested by using 0.05% (w/v) trypsin and 0.02% (w/v) EDTA, pooled and seeded at a ratio of 1:5 into 100 mm diameter Petri dishes. Cells for the experiments described were derived from passages 2–4 of individual preparations. They were identified after fixation with *p*-formaldehyde by positive immunostaining for smooth muscle  $\alpha$ -actin with a monoclonal anti- $\alpha$ -smooth muscle actin clone from mouse ascites (dilution 1:1000, Sigma-Aldrich, Deisenhofen, Germany), a secondary anti-mouse IgG-FITC conjugate from sheep (dilution 1:60, Sigma-Aldrich) and visualization of the actin fibers by confocal laser scanning microscopy (Leica Microsystems, Heidelberg, Germany). According to this procedure, the cultured raSMC appeared to be essentially homogenous and free of fibroblasts.

All incubation were performed in Waymouth medium containing

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10% FBS with smooth muscle cells grown to >80% confluence either in six well plates ( $1.5 \times 10^6$  cells/well) for reverse transcription (RT)-PCR analyses or in 60 mm diameter Petri dishes ( $5 \times 10^6$  cells/dish) for electrophoretic mobility shift analyses (EMSA) and Western blot analyses. Incubations were terminated by rinsing the cells with Hank's balanced salt solution followed by mRNA or protein extraction. The cell viability was assessed at the light microscope level as well as by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay [14] and found to be largely unaffected by any of the test compounds studied.

## 2.2. RT-PCR analysis

The cultured cells were lysed with guanidinium thiocyanate (GTC) buffer (Qiagen, Hilden, Germany) and total RNA was extracted with the Qiagen RNeasy kit. First strand cDNA synthesis from 3  $\mu$ g of total RNA was performed with Superscript reverse transcriptase (Life Technologies) according to the manufacturer's instructions. To normalize the cDNA amount in the samples, 2.5% of the resulting cDNA was used for PCR analysis of the house keeping genes, elongation factor 2 (EF-2) or glyceraldehydephosphate dehydrogenase (GAPDH). PCR reactions were performed with as few cycles as possible to detect the PCR products in ethidium bromide (0.1%) stained 1.5% agarose gels and cDNA volumes were adjusted for consecutive analyses by densitometry (One-Dscan Gel analysis software from Scanalytics, Billerica, MA, USA). Programs and primers for the measurement of steady state levels of mRNA were as follows.

A unique 2 min period for complete denaturation at 94°C in the beginning, followed by a primer-specific number of cycles of 30 s denaturation at 94°C, 30 s annealing at 58°C (CD40 54°C, CD154 55°C) and 1 min primer extension at 72°C, with an additional 5 min at 72°C for the final extension in the end. CD40: expected size 442 bp, 26–32 cycles, sense 5'-GTGTGTTACGTGCAGTGACAA-3', anti-sense 5'-ATCCTCACAGCTTGTTCA 3' (corresponding to nucleotide sequences 74–94 and 515–498 of the murine CD40 gene, GenBank accession number M83312); CD154: 435 bp, 26–37 cycles, sense 5'-TTTTGCTGTGTATCTTCATAG-3', anti-sense 5'-CGTTGAC-TCGAAGGCTCCCGA-3' (135–155 and 569–549, murine CD154 gene, X65453); EF-2: 218 bp, 19–26 cycles, sense 5'-GACATCAC-CAAGGTGTGCAG-3', anti-sense 5'-GCGGTCAGCACAATGG-CATA-3' (1990–2010 and 2207–2188, human EF-2 gene, Z11692); GAPDH: 572 bp, 20–24 cycles, sense 5'-TCACCATCTTCCAG-GAGCG-3': anti-sense 5'-CTGCTTACCACCTCTTGA-3' (245–263 and 816–797, rat GAPDH gene, M17701).

All PCR reactions were performed in a Hybaid Omne thermocycler (AWG, Heidelberg, Germany). To verify the identity of the amplification products with the designed primer pairs, we cloned and sequenced the CD40 and CD154 PCR products and found a 87 and 93% homology with the published sequences of the murine genes, respectively.

## 2.3. EMSA

Nuclear extracts from the cultured raSMC were prepared essentially as described [15] except that Igepal CA-630 (Sigma-Aldrich) was used instead of Nonidet-P40 as a detergent. Aliquots of nuclear protein (10  $\mu$ g) were incubated with 10–20000 cpm of the double-stranded (ds) oligodeoxynucleotides (ODN) in binding buffer [16] containing 1.33 mmol/l DTT and 1  $\mu$ g poly(d(I-C)) in a total volume of 15  $\mu$ l for 30 min at ambient temperature. The resulting protein-DNA complexes were analyzed by non-denaturing polyacrylamide gel (4%) electrophoresis and autoradiography by exposing the dried gels to Kodak X-OMAT AR X-ray film (Sigma-Aldrich). To monitor the specificity of the binding reaction, supershift analyses were performed by pre-incubation of the nuclear extracts (10  $\mu$ g protein) with 2  $\mu$ g of a polyclonal rabbit anti-nuclear factor (NF)- $\kappa$ B p65 or rabbit anti-signal transducer and activator of transcription (Stat)-1 $\alpha$  p91 antibody (Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at ambient temperature in a total volume of 6  $\mu$ l. The consensus ODN (Santa Cruz Biotechnology) were labelled with  $^{32}$ P by using the 5'-end labelling kit from Amersham Pharmacia Biotech (Freiburg, Germany).

## 2.4. Decoy ODN technique

Ds decoy ODN were prepared from the complementary single-stranded (ss) phosphorothioate-bonded ODN (Eurogentec, Cologne, Germany) by melting at 95°C for 5 min followed by a cool-down phase of 3 h at ambient temperature. The efficiency of the hybrid-

ization reaction was checked on 2.5% agarose gels containing 0.1% ethidium bromide and usually found to exceed 80%. The ds decoy ODN were pre-incubated with the cultured raSMC for 4–16 h at concentrations ranging from 4 to 16  $\mu$ M. Thereafter, the decoy ODN-containing medium was replaced by fresh medium to which the various test compounds were added. On the basis of subsequent EMSA and RT-PCR analyses, an optimum concentration of 10  $\mu$ M and a pre-incubation period of 4 h was determined for all decoy ODN used (not shown). The ss sequences of the decoy ODN were as follows (underlined letters denote phosphorothioate-bonded bases): activator protein-1 (AP-1, sense 5'-CGCTTGATGACTCAGCCGGAA-3'; CCAAT enhancer binding protein (C/EBP), palindrome 5'-TGCA-GATTGCGCAATCTGCA-3'; interferon regulatory factor-1 (IRF-1), sense 5'-GGAAGCGAAAATGAAATTGAC-3'; NF- $\kappa$ B, sense 5'-AGTTGAGGGGACTTCCAGG-3'; Stat-1, sense 5'-CATGT-TATGCATATTCCTGTAAGTG-3'.

## 2.5. Nuclear run-on analysis

All steps were performed at 0–4°C. Approximately  $5 \times 10^6$  raSMC in 100 mm Petri dishes were exposed to interleukin-1 $\beta$  (IL-1 $\beta$ , 60 U/ml) plus tumor necrosis factor  $\alpha$  (TNF $\alpha$ , 1000 U/ml) or vehicle for 3 h. Thereafter, the cells harvested with a cell scraper and incubated for 10 min in two volumes of lysis buffer (5 mM HEPES, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 20% glycerol, 0.05% Triton X-100 pH 7.4) after which time the volume was adjusted to 2 ml with cold wash buffer (20 mM Tris, 20% (w/v) glycerol, 140 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT, protease inhibitor mix pH 7.4) and the cells were lysed with 15 strokes in a EMBL cell cracker device as described [17]. The lysate was centrifuged for 20 s at 300 $\times$ g and 0–4°C in a micro-centrifuge, the pellet discarded and the supernatant centrifuged for 5 min at 1650 $\times$ g. The nuclear pellet was washed three times with 1 ml cold wash buffer, collected by centrifugation at 1650 $\times$ g for 5 min and suspended in approximately four volumes of reaction buffer (2 mM each CTP, GTP and UTP, 3 mM ATP, 20 U/ml RNasin (Fermentas, Vilnius, Lithuania), 8.5 mM creatinephosphate and 0.1 mg/ml creatine kinase (Roche Diagnostics, Mannheim, Germany) in cold wash buffer). Half of the nuclei were immediately lysed in four volumes of GTC buffer as the negative control and the other half was incubated for 30 min at 30°C and then lysed. Total RNA was isolated and RT-PCR performed as described before, except for the use of random primers instead of oligo-dT primers in the reverse transcription step. When RT-PCR with mRNA from control nuclei was performed, the amount of cDNA synthesized was typically not detectable or amounted to maximally 20% of that from the incubated nuclei, demonstrating that the PCR products obtained from this procedure reflected de novo mRNA synthesis in the isolated nuclei.

## 2.6. Western blot analysis

Western blot analysis of CD40 protein expression in the raSMC (cultured in 60 mm diameter dishes, confluence >80%) was essentially performed as described [18] except that cell homogenates were additionally treated for 15 min with 0.5% (w/v) CHAPS at 0–4°C to enrich the membrane-bound CD40 protein in the 10000 $\times$ g supernatant. Positive control samples from rat thymus and spleen were prepared in essentially the same manner, except that the tissue was homogenized at 0–4°C by using a glass potter with a teflon piston. Protein concentrations were determined by using the sodium dodecyl-sulfate (SDS) modification of the micro-Lowry method [19]. Aliquots of the protein extracts (10–50  $\mu$ g/lane) were separated by denaturing polyacrylamide gel (10%) electrophoresis in the presence of SDS and then transferred onto nitrocellulose membranes. The immobilized CD40 protein was visualized by subsequent incubation with a polyclonal rabbit anti-CD40 antibody (1:2500 dilution in blocking buffer, Research Diagnostics, Flanders, NJ, USA) for 2 h at ambient temperature followed by a polyclonal peroxidase-conjugated anti-rabbit antibody (1:7500 dilution, Sigma-Aldrich) for 1 h at ambient temperature, incubation with the Super Signal Blaz chemiluminescence reagent according to the manufacturer's instructions (Pierce Chemical, Rockford, IL, USA) and exposure to Kodak X-OMAT AR film for up to 5 min. Thereafter, the loading and transfer of equal amounts of protein in each lane was verified by staining of the protein bands on the nitrocellulose membrane with indian ink followed by densitometry.

## 2.7. Statistical analysis

Unless indicated otherwise, all data in the figures and text are ex-

pressed as mean  $\pm$  S.E.M. of  $n$  observations. Statistical evaluation was performed by Student's  $t$ -test for unpaired data with the Instat for Windows statistics software package (GraphPad Software, San Diego, CA, USA) and a  $P$  value  $< 0.05$  considered statistically significant.

### 3. Results

#### 3.1. CD40 and CD154 expression

In the cultured raSMC, no expression of CD154 could be verified at the mRNA level in the absence or presence of interferon- $\gamma$  (IFN $\gamma$ ) plus TNF $\alpha$ . The same was true for vascular SMC enzymatically isolated from the rat mesentery and the endothelium intact rat aorta (i.e. native endothelial and smooth muscle cells, Fig. 1a). Following exposure to IFN $\gamma$  plus TNF $\alpha$ , on the other hand, there was a distinct expression of CD40 in the cultured SMC as well as in the aorta (Fig. 1a). IFN $\gamma$  and TNF $\alpha$  alone elicited only a moderate increase in CD40 mRNA expression, but when combined, produced a

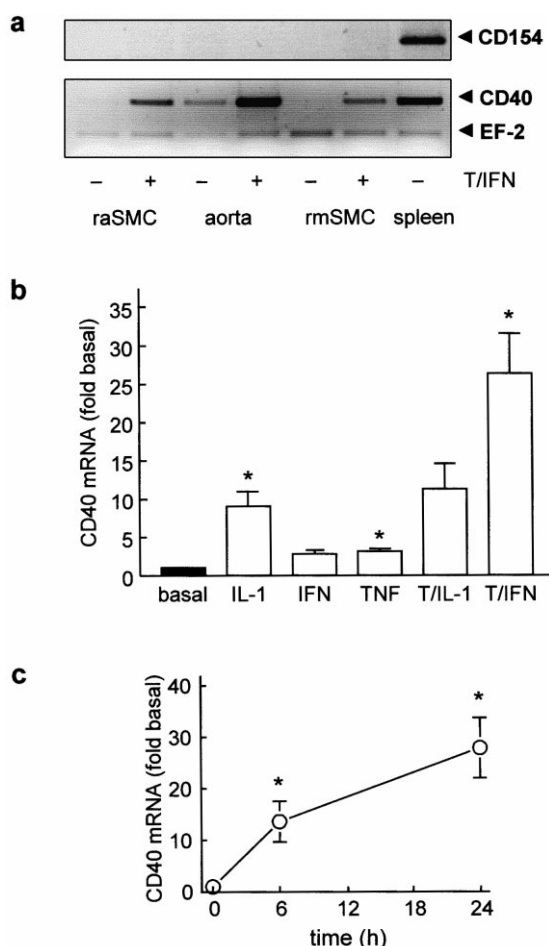


Fig. 1. (a) Expression of CD40 (30 cycles) and CD154 (34 cycles mRNA) in non-stimulated and TNF $\alpha$  (1000 U/ml) plus IFN $\gamma$  (100 U/ml)-stimulated (T/IFN) cultured raSMC, cultured mesenteric SMC (rmSMC), endothelium-intact rat aorta and rat spleen. (b) Effects of IL-1 $\beta$  (IL-1, 60 U/ml), IFN $\gamma$  (IFN, 100 U/ml), TNF $\alpha$  (TNF, 1000 U/ml), TNF plus IL-1 $\beta$  (T/IL-1) and TNF $\alpha$  plus IFN $\gamma$  (T/IFN) on CD40 mRNA expression after 24 h (expressed as multiple of the level in non-stimulated cells) in cultured raSMC ( $n=3-7$ , \* $P<0.05$  versus basal). (c) Time-dependent effect of TNF $\alpha$  plus IFN $\gamma$  on CD40 mRNA expression in cultured raSMC ( $n=6-7$ , \* $P<0.05$  versus basal).

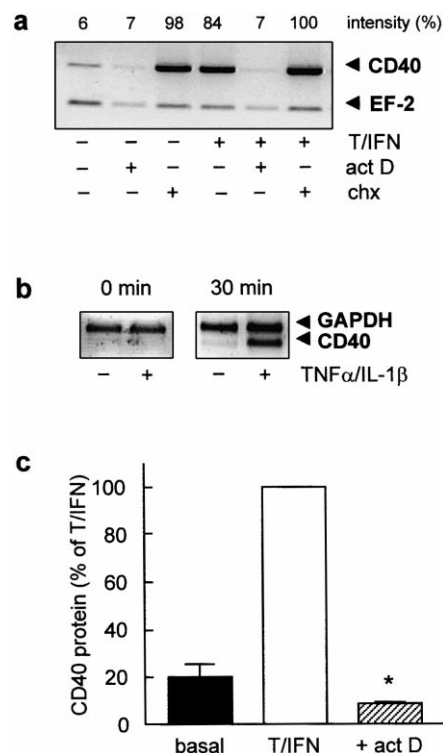


Fig. 2. (a) Effects of actinomycin D (act D, 1  $\mu$ M) and cycloheximide (chx, 1  $\mu$ M) on CD40 mRNA expression in cultured raSMC after 24 h under basal conditions and following stimulation with TNF $\alpha$  (1000 U/ml) plus IFN $\gamma$  (100 U/ml, T/IFN). The figure depicts a typical RT-PCR analysis with the relative intensities (%), as judged by densitometry, indicated at the top. Comparable findings were obtained in two further experiments with different batches of raSMC. (b) PCR-based run-on analysis of the de novo expression of CD40 and GAPDH mRNA in isolated nuclei of raSMC exposed to 1000 U/ml TNF $\alpha$  plus 60 U/ml IL-1 $\beta$  (+) or vehicle (–) incubated for 30 min or lysed immediately after the isolation (0 min). The figure depicts a typical run-on analysis, qualitatively identical results were obtained in two further experiments with different batches of raSMC. (c) Effect of actinomycin D (+act D, 1  $\mu$ M) on the TNF $\alpha$  (1000 U/ml) plus IFN $\gamma$  (100 U/ml)-stimulated (T/IFN) increase in CD40 protein abundance (expressed as percentage of the maximal value) in cultured raSMC after 24 h ( $n=4-7$ , \* $P<0.05$  versus T/IFN).

marked (Fig. 1b) and time-dependent (Fig. 1c) synergistic effect. Combination of these two cytokines also significantly enhanced CD40 protein expression (cf. Fig. 2c). IL-1 $\beta$  alone also elicited a substantial increase in CD40 mRNA expression which was significantly more pronounced than that caused by either IFN $\gamma$  or TNF $\alpha$  alone. When combined with TNF $\alpha$ , on the other hand, IL-1 $\beta$  revealed only an additive effect (Fig. 1b).

#### 3.2. Transcription factor activation

This cytokine-inducible CD40 expression was abolished by co-incubation with actinomycin D while cycloheximide markedly enhanced basal CD40 mRNA abundance (Fig. 2a). Co-incubation with actinomycin D also abrogated the cytokine-induced increase in CD40 protein (Fig. 2c) and subsequent run-on analyses confirmed that the cytokine-induced increase in CD40 expression in the cultured raSMC is indeed regulated at the transcriptional level (Fig. 2b).

Therefore, cytokine-inducible transcription factor activation in these cells was analyzed by EMSA. As the promoter region

of the rat CD40 gene has not been sequenced yet, we employed consensus ODN together with the appropriate super-shift antibodies. In nuclear extracts of raSMC exposed to IFN $\gamma$  plus TNF $\alpha$ , a significant and prolonged translocation of the p65/p50 heterodimer (RelA) of NF- $\kappa$ B as well as of Stat-1 (presumably the p91/p91 homodimer, i.e. Stat-1 $\alpha$ ) to the nucleus was detected as early as 30 min (Fig. 3, top panel). Addition of the NF- $\kappa$ B inhibitor, pyrrolidine dithiocarbamate (PDTC, 100  $\mu$ M/l), to the raSMC 1 h prior to that of the cytokines not only attenuated the activation of NF- $\kappa$ B, but also that of Stat-1 (Fig. 3, bottom panel). In contrast, no significant change in the activity of the transcription factors AP-1, C/EBP and IRF-1 was observed in the presence of both cytokines (not shown).

### 3.3. Effects of decoy ODN

To confirm the involvement of NF- $\kappa$ B and Stat-1 in cytokine-inducible CD40 expression, the decoy ODN technique was employed. Both cytokine-stimulated CD40 mRNA (Fig. 4a) and protein expression (Fig. 4b) were significantly reduced following pre-treatment of the raSMC with an NF- $\kappa$ B- or Stat-1-specific decoy ODN to approximately 50% of the control value. According to EMSA, the decoy ODN used were highly specific for their target transcription factor, the abundance of which was significantly reduced in nuclear extracts prepared from decoy ODN-treated cells, while that of the other transcription factors was not affected (not shown).

Moreover, neither the consensus decoy ODN against C/EBP nor those against AP-1 or IRF-1 had a significant effect on CD40 mRNA expression both in quiescent and cytokine-stimulated raSMC, while both the AP-1 and the C/EBP decoy

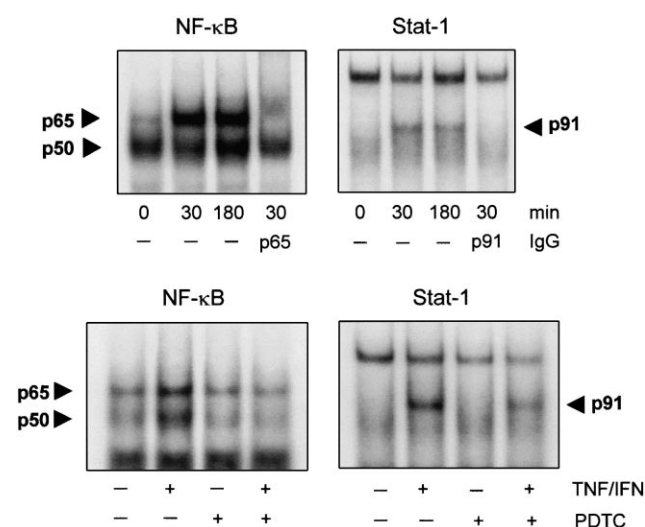


Fig. 3. (Top panel) Time-dependent changes in the abundance of NF- $\kappa$ B (p65/p50 heterodimer and p50/p50 homodimer) and presumably the p91/p91 homodimer of Stat-1 in the nuclei of TNF $\alpha$  (1000 U/ml) plus IFN $\gamma$  (100 U/ml)-stimulated (TNF/IFN) cultured raSMC. The figure depicts typical EMSA with the appropriate supershift analysis performed with a single batch of raSMC. Identical findings were obtained with at least three further batches of raSMC for each transcription factor. (Bottom panel) Effects of PDTC (100  $\mu$ M) on basal and TNF $\alpha$  plus IFN $\gamma$ -stimulated (TNF/IFN) nuclear translocation of NF- $\kappa$ B and Stat-1 in cultured raSMC after a 30 min incubation. The figure depicts two typical EMSA performed with the same batch of cells. Comparable findings were obtained for each transcription factor in at least two further experiments with different batches of raSMC.

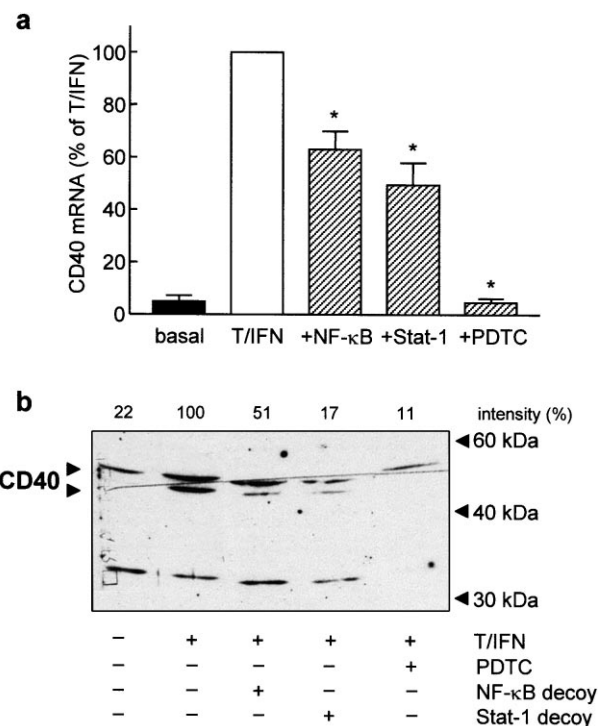


Fig. 4. (a) Effects of 100  $\mu$ M PDTC (1 h pre-incubation) and 10  $\mu$ M each of the indicated consensus decoy ODN (4 h pre-incubation) on TNF $\alpha$  (1000 U/ml) plus IFN $\gamma$  (100 U/ml)-stimulated (T/IFN) CD40 mRNA expression (expressed as percentage of the maximal value) in cultured raSMC after 6 h ( $n=4-6$ , \* $P<0.05$  versus T/IFN). (b) Original Western blot showing the effect of PDTC and that of the NF- $\kappa$ B and Stat-1 consensus decoy ODN on CD40 protein expression in TNF/IFN-stimulated raSMC. Experimental conditions were as described above, except that incubations were terminated after 24 h. The relative intensities of the two CD40-specific protein bands (judged by densitometry and expressed as percentage of the maximal level) are indicated at the top of the figure. Comparable findings were obtained in two further experiments with different batches of raSMC.

ODN clearly attenuated the cytokine-induced expression of other gene products in these cells (not shown). Compatible inhibitory effects of the decoy ODN for NF- $\kappa$ B and Stat-1 on CD40 mRNA expression were also observed with human umbilical vein cultured endothelial cells exposed to TNF $\alpha$  plus IFN $\gamma$  (not shown).

## 4. Discussion

Due to the nature of the interaction between CD40 receptor and ligand resulting in a trimerization of the receptor protein [2,3], antibodies against CD40 tend to activate the CD40 signalling pathway in CD154 target cells rather than blocking it. Antagonists for CD40 are also not yet available so that apart from CD154-blocking antibodies, targeting of CD40 gene expression (i.e. by suppressing the activation of essential transcription factors) may provide a means for interfering with the CD40-CD154-mediated interaction of activated T-cells and the vessel wall, a consideration that prompted us to investigate in more detail cytokine-inducible CD40 gene expression in raSMC.

With respect to the cytokines capable of inducing CD40 expression, we could confirm both at the mRNA and protein

level previous findings in human cultured endothelial [7,8] and smooth muscle cells [4] of an up-regulation of CD40 immunoreactivity following exposure to IFN $\gamma$ , IL-1 $\beta$ , TNF $\alpha$  and especially to the combination of IFN $\gamma$  and TNF $\alpha$ . Of note was that we routinely detected two CD40 immunoreactive bands by Western blot analysis both in the positive control (rat thymus) and in the cultured raSMC, a major 48 kDa band and a minor band migrating at 45 kDa. The identity of the second band is unclear at present, but it may represent a differently glycosylated or phosphorylated form of the membrane-bound CD40 or the soluble isoform [2].

Both basal and cytokine-stimulated CD40 expression in the cultured raSMC was either abolished or markedly attenuated in the presence of the RNA synthesis inhibitor, actinomycin D, hence pointing to a *de novo* expression of the CD40 gene, a notion that was verified by subsequent nuclear run-on analyses. Especially basal CD40 mRNA expression, on the other hand, was markedly up-regulated following co-incubation with the protein synthesis inhibitor, cycloheximide. While this is often assumed to result from mRNA stabilization by cycloheximide, another explanation is that CD40 is a primary response gene, the expression of which is regulated by cycloheximide-sensitive transcription factors. NF- $\kappa$ B is a prime candidate for such a transcription factor, as its activity is enhanced due to the fact that cycloheximide prevents the rapid NF- $\kappa$ B-induced re-synthesis of the inhibitory I $\kappa$ B $\alpha$  subunit [20].

Prompted by this finding, we analyzed five different transcription factors (AP-1, C/EBP, IRF-1, NF- $\kappa$ B and Stat-1) that we thought might be involved in cytokine-inducible CD40 gene expression. These transcription factors have either been shown previously to play an important role in the expression of other primary response genes [20–23] or were known to be preferably activated by IFN $\gamma$  [24,25]. According to EMSA, only two transcription factors, the p65/p50 heterodimer of NF- $\kappa$ B (RelA) and presumably the p91/p91 homodimer of Stat-1 (Stat-1 $\alpha$ ), were rapidly translocated to the nucleus of the stimulated raSMC. Interestingly, the NF- $\kappa$ B inhibitor, PDTC, not only inhibited the cytokine-stimulated translocation of NF- $\kappa$ B, but also that of Stat-1. Moreover, PDTC virtually abolished the IFN $\gamma$ /TNF $\alpha$ -induced expression of CD40, suggesting that these two transcription factors indeed play a crucial role in IFN $\gamma$ /TNF $\alpha$ -stimulated CD40 gene expression in these cells.

To further substantiate this hypothesis, the decoy ODN technique was employed by using the same consensus ODN as for the EMSA but modified with phosphorothioate for added stability. After thoroughly testing their target specificity, optimum concentration and pre-incubation period by EMSA, IFN $\gamma$ /TNF $\alpha$ -mediated CD40 mRNA and protein expression were found to be significantly down-regulated following exposure to the decoy ODN for NF- $\kappa$ B and Stat-1, but not by those directed against AP-1, C/EBP or IRF-1.

When considering the PDTC, EMSA and decoy ODN data together, they all suggest that IFN $\gamma$  via activation of Stat-1 $\alpha$  and TNF $\alpha$  via activation of NF- $\kappa$ B synergistically induce CD40 gene expression in the cultured raSMC. This most likely holds true also for CD40-expressing human cells. Thus, a role for NF- $\kappa$ B in CD40 expression has recently been proposed for Burkitt lymphoma cells stimulated with Epstein-Barr virus latent membrane protein-1 [26]. Moreover, the comparable inhibitory effect of the NF- $\kappa$ B and Stat-1

decoy ODN on IFN $\gamma$ /TNF $\alpha$ -stimulated CD40 expression in human cultured endothelial cells (M. Hecker and A. Wagner, unpublished observation) as well as the putative binding sites for transcription factors that can be identified in the 5'-flanking region of the human CD40 gene also substantiate this conclusion.

Thus far, 758 bases of the 5'-flanking region of the human CD40 gene have been sequenced (submitted to GenBank in 1995, accession number X87626), but transcription factor binding sites have not been analyzed in detail. We therefore analyzed the 5'-flanking region of the human CD40 gene again by using the MatInspector V2.2 software package [27] and identified three NF- $\kappa$ B p65 binding sites at –183, –291 and –587 bases and two Stat sites at –228 and –570 bases. The 5'-flanking region of the human CD40 gene, however, does not contain a classical TATA box or binding sites for AP-1, C/EBP or IRF-1 when scanned with the appropriate stringency and this would fit to the observed lack of effect of the corresponding decoy ODN on cytokine-stimulated CD40 expression in the cultured raSMC.

What are the functional consequences of this cytokine-induced increase in CD40 expression in vascular SMC? Interestingly, we were unable to detect any expression of CD154 both in native and cultured SMC from the rat. This finding is at variance with a recent report stating that functional CD154 is expressed on human endothelial and smooth muscle cells and is up-regulated by IFN $\gamma$ , IL-1 $\beta$  or TNF $\alpha$  during arteriosclerosis [4]. We do not yet have a plausible explanation for these conflicting data, but in our hands, both human and rat endothelial and smooth muscle cells act as true recipients for CD154, i.e. they do not express the ligand themselves even in the presence of the afore-mentioned cytokines. One interesting suggestion has been that the interaction of T-cells and vascular SMC via CD40-CD154 triggers the expression of matrix-degrading enzymes in the SMC that promote plaque destabilization or rupture [28]. In a similar context, the interaction between CD154-expressing platelets [5] and CD40-expressing SMC may be of relevance for restenosis after angioplasty and poses the intriguing question whether activation of vascular SMC via CD40 promotes apoptosis or proliferation of these cells.

In summary, the present findings demonstrate that the marked synergistic effect of the pro-inflammatory cytokines, IFN $\gamma$  and TNF $\alpha$ , on CD40 gene expression in raSMC (and most likely also in human cultured endothelial cells) occurs at the level of transcription and is mediated by the simultaneous activation of NF- $\kappa$ B/RelA by TNF $\alpha$  and Stat-1 $\alpha$  by IFN $\gamma$ . The potent suppression by the decoy ODN of Stat-1-dependent CD40 expression may provide an interesting therapeutic tool for specifically interfering with CD40-CD154-mediated chronic inflammatory responses *in vivo* including arteriosclerosis, as both the activation of CD154-expressing T-lymphocytes [29,30] and the expression of CD40 in endothelial and smooth muscle cells [4,7,8] appears to be IFN $\gamma$ -dependent.

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